# Attenuation of Oxidant-induced Lung Injury by 21-Aminosteroids (Lazaroids): Correlation with the mRNA Expression for E-selectin, P-selectin, ICAM-1, and VCAM-1

Robert L. Griffin,[1] Raymond F. Krzesicki,[1] Stephen F. Fidler,[1] Craig L. Rosenbloom,[2] John A. Auchampach,[2] Anthony M. Manning,[2] Joseph V. Haas,[3] Sue K. Cammarata,[4] Jia En Chin,[1] and Ivan M. Richards[1]

[1]Hypersensitivity Diseases Research, Upjohn Laboratories, Kalamazoo, Michigan; [2]Adhesion Biology, Upjohn Laboratories, Kalamazoo, Michigan; [3]Research Support Biostatistics, Upjohn Laboratories, Kalamazoo, Michigan; [4]Borgess Hospital, Kalamazoo, Michigan

We compared the effects of treatment with methylprednisolone or the 21-aminosteroids, U-74389 and U-74006F (Tirilizad mesylate), on hyperoxic lung injury and the associated expression of mRNA for several adhesion molecules in rats. Inhalation of >95% oxygen for up to 72 hr in Sprague–Dawley rats produced a marked increase in lung weight and an accumulation of fluid in the thorax when compared with air-breathing controls. Hyperoxia also induced a marked neutrophil-rich influx of inflammatory cells into the bronchial lumen as measured by bronchoalveolar lavage. Neutrophil numbers in bronchoalveolar lavage fluid peaked after 60 hr of exposure to >95% oxygen; this was associated with a marked upregulation of mRNA for the adhesion molecules P-selectin and E-selectin but not VCAM-1. mRNA for ICAM-1 was constitutively expressed at high levels in both air-breathing controls and in the lungs of rats exposed to high concentrations of oxygen. Pretreatment with the 21-aminosteroids reduced hyperoxic lung damage and improved survival times in animals exposed to >95% oxygen. However, treatment with methylprednisolone significantly decreased survival times. Treatment with U-74389 did not significantly ($p > 0.05$) inhibit the BAL neutrophilia and did not significantly ($p > 0.05$) reduce hyperoxia-induced increases in mRNA expression for P-selectin and E-selectin. The inhibition of hyperoxic lung damage coupled with improved survival seen in treated animals suggests that 21-aminosteroids may provide valuable treatments for pulmonary disorders in which oxidant damage has been implicated. — Environ Health Perspect 102(Suppl 10):193–200 (1994)

Key words: hyperoxic lung injury, hyperoxia, P-selectin, E-selectin, ICAM-1, VCAM-1, 21-aminosteroids

## Introduction

Although oxygen therapy has proved invaluable in infants, children, and adults with respiratory insufficiency, prolonged exposure to oxygen can lead to lung cell damage, lung fibrosis, bronchopulmonary dysplasia, organ dysfunction, and death from pulmonary edema (*1*). Presently, there is no commonly accepted pharmacologic treatment for patients suffering from the harmful effects of hyperoxia. In addition to hyperoxic lung injury, a number of clinical and experimental pulmonary disorders have been associated with increased oxidant stress, where the local antioxidant defenses in the lungs are overwhelmed by the oxidant burden arising from diverse sources. These include ischemia followed by tissue reoxygenation, the administration of toxins that augment intracellular oxidant formation, and the accumulation of activated leukocytes in the lung tissues. Clinical pulmonary disorders in which oxidant damage has been implicated include adult respiratory distress syndrome (ARDS) (*2–4*), idiopathic pulmonary fibrosis (*5,6*), cystic fibrosis (*7–9*), emphysema (*10,11*), and asthma (*12,13*).

Modification of the basic structure of methylprednisolone led to the introduction of a new class of compounds, 21-aminosteroids or "first-generation" lazaroids, originally designed as nonglucocorticoid, iron-dependent inhibitors of lipid peroxidation (*14,15*). Members of this class of compound have greater potency than methylprednisolone in animal models of central nervous system trauma and neuronal membrane damage (*16,17*). Close structural analogs also inhibit antigen-induced lung eosinophilia in animals (*18*) and inhibit oxidant-mediated injury in isolated rat lungs after ischemia/reperfusion or after *t*-butylhydroperoxide challenge (*19*). It would be of obvious interest to explore the potential of these compounds in lung diseases in which oxidant damage has been implicated. In this article, we describe the attenuation of hyperoxic lung injury in rats following treatment with the 21-aminosteroids, U-74389* (21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-sulfonate (Figure 1) or U-74006F (21-[4- (2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione monomethane sulfonate (Figure 1), but not following treatment with methylprednisolone. We also compared the effects of treatment with methylprednisolone or the 21-aminosteroids on survival times of rats held in a hyperoxic environment for up to 96 hr.

This paper was presented at the Conference on Oxygen Radicals and Lung Injury held 30 August–2 September 1993 in Morgantown, West Virginia.

We thank Dr. E. D. Hall of Upjohn Laboratories for his helpful advice and discussions concerning the mechanism of action of 21-aminosteroids.

Address correspondence to Ivan M. Richards, Hypersensitivity Diseases Research, Upjohn Laboratories, 301 Henrietta Street, Kalamazoo, MI 49001. Telephone (616) 385-7040. Fax (616) 384-9324.

*U-74389 was tested as either the methane sulfonic acid (U-74389F) or maleic acid (U-74389G) salts.

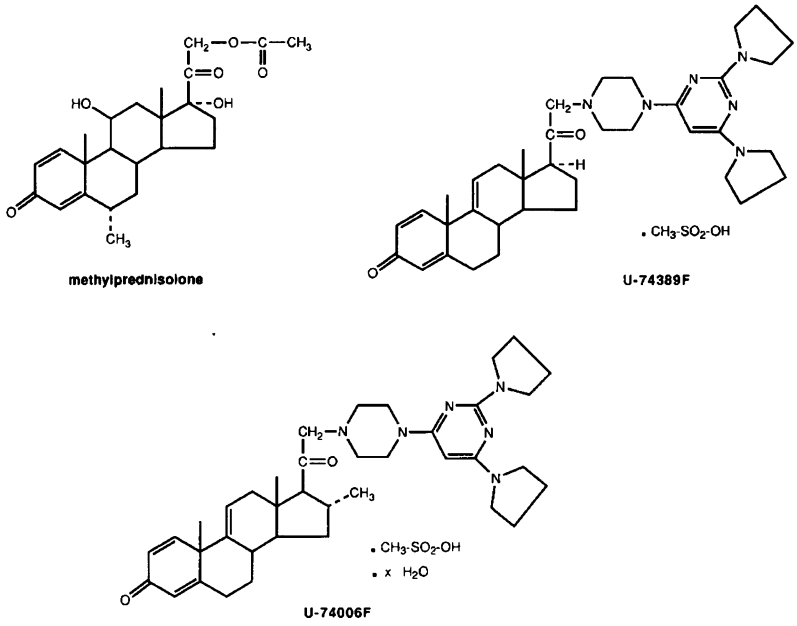


**Figure 1.** Chemical structures of methylprednisolone and the nonglucocorticoid 21-aminosteroids U-74389G and U-74006F.

The role of the neutrophil in contributing to hyperoxic lung injury is controversial, although it is well established that the neutrophil burden in the lungs is markedly increased after exposure to oxygen for 36 hr or more (*20*). The recruitment of neutrophils into inflamed lung tissue involves multiple adhesion molecules and several discrete steps, including rolling of the cells on the activated endothelial cells, firm adhesion to and migration through the endothelial cells of the blood vessel wall into the surrounding tissues, and in some cases a further migration of the cells through the airway epithelium into the bronchial lumen (*21–28*).

In the present experiments we attempted to correlate the time course of neutrophil infiltration into rat lungs during exposure to >95% oxygen, with the expression in lung tissues of mRNA, by Northern blot analysis, for the adhesion molecules, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1; E-selectin), and intercellular adhesion molecule-1 (ICAM-1). We also investigated the effects of treating rats with the 21-aminosteroid U-74389F on the hyperoxia-induced lung neutrophilia and on the expression of mRNA for the above adhesion molecules during hyperoxic lung injury.

## Methods

### Induction and Time Course of Hyperoxic Lung Injury in Rats

Groups of 6 to 12 male Sprague–Dawley rats, 225 to 250 g, were housed in clear 40-l plexiglass exposure chambers for the duration of the period of oxygen exposure. The animals were supported on a stainless steel mesh grid and were allowed food and water *ad libitum*. The chambers were cleaned twice daily and were equipped with separate air-tight openings to allow feeding, cleaning, and monitoring oxygen concentrations. One hundred percent oxygen was delivered and exhausted through ports at approximately five to seven volume changes per hour to maintain a concentration of >95% oxygen in the chambers. After exposure to >95% oxygen or air for 24, 48, 60, or 72 hr, the animals were killed by an overdose of urethane, ip, the lungs were removed, weighed, dried overnight in an oven, and reweighed. The volume of fluid accumulating in the thorax was also measured.

Animals were dosed, ip, 1 hr prior to oxygen exposure and then twice daily throughout the period of oxygen exposure with vehicle (CS4), U-74389F, U-74006F, or methylprednisolone. At various time points after oxygen exposure, the rats were killed with an overdose of urethane.

### Bronchoalveolar Lavage

At 24, 48, 60, and 72 hr after exposure to >95% oxygen, each rat was anesthetized with 1.5 g/kg urethane, ip, the trachea was cannulated and 5.0 ml of PBS was instilled into the lungs. The tracheal cannula was clamped and the thorax was massaged for 30 sec before recovering the bronchoalveolar lavage (BAL) fluid. A further 5 ml PBS was then instilled into the lungs, and the procedure was repeated. Ten milliliters of Hank's balanced salt solution (HBSS) containing 5% fetal calf serum was then added to the pooled and recovered lavage fluid and vortexed. After centrifugation (150*g*, 10 min, 4°C) the supernatant was discarded and the cells were resuspended in 5.0 ml HBSS containing 5% fetal calf serum. For cytologic examination, cytospin preparations were made using a Shandon cytocentrifuge (150*g*, 10 min, room temperature) (Shandon Southern Instruments, Sewickley, PA). The cells were fixed and stained using Diff QuikR (American Scientific Products, McGraw Park, IL). Differential cell counts on at least 100 cells were made using standard morphologic criteria to classify the cells into eosinophils, neutrophils, and mononuclear cells.

### Preparation of cDNA Probes

cDNA clones encoding rat P-selectin and E-selectin were isolated by homology cloning (Manning et al., manuscript in preparation). A cDNA clone encoding rat ICAM-1 (*29*) was kindly provided by Tadashi Horiuchi (Daiichi Pharmaceutical Co., Tokyo, Japan). A rat VCAM-1 cDNA fragment was isolated by polymerase chain reaction (PCR) from first-strand cDNA synthesized from total RNA isolated from the heart tissue of rats 3 hr after administration of bacterial endotoxin. Gene-specific oligonucleotides were designed based on the available cDNA sequence for rat VCAM-1 (*30*). The resulting PCR product was subcloned and its authenticity confirmed by DNA sequence analysis.

For use in hybridization protocols, DNA fragments were prepared from these cDNA clones by the PCR. Gene-specific oligonucleotides were designed to generate fragments of approximately 500 bp in length from the P-selectin, VCAM-1, and ICAM-1 cDNA clones, and 1.6 Kb in length from the rat E-selectin cDNA clone. The following oligonucleotide pairs were used (5′oligo/3′oligo; each sequence as 5′ to 3′): P-selectin CGACTTGACTGTCACTCA/ACAAGTGAGATACACAG encompassing sequences encoding the cytoplasmic domain and 3′ untranslated region; E-selectin TTACTACTGGATTGGAATCAG/TGTTTCTGATTGTTTTGAACTTA encompassing sequences encoding the EGF-like, complement regulatory-like repeats, the transmembrane and cytoplasmic domains; ICAM-1 AGGTGTGATATCCGGTAGA/CCTTCTAAGTGGTTGGAACA encompassing the

3'untranslated region; and VCAM-1 CCAAGCTATGCATTCAGACT/CTGAAAGTCAACCCAGTGAC encompassing the 3' untranslated region.

### RNA Extraction and Northern Blot Analysis

Total rat lung RNA was isolated using the RNAzol (Cinna/Biotecx Laboratories International, Inc., Friendswood, TX) method, which is a modification of the Chomczynski and Sacchi (*31*) single step procedure. Equivalent amounts of RNA (10 μg/lane) were denatured with glyoxal and DMSO and applied to 1.5% agarose gels. The RNA was transferred to Nylon 66 Plus membranes (Hoeffer Scientific, San Francisco, CA) by vacuum blotting and UV cross-linked. The blots were prehybridized in hybridization buffer (1% BSA, 1 mM EDTA, and 7% SDS in 0.5 M $NaHPO_4$, pH 7.2) for 60 min and hybridized overnight at 65°C. The blots were then washed twice with 0.5% BSA, 1 mM EDTA, and 5% SDS in 40 mM $NaHPO_4$, pH 7.2, and four times with 1 mM EDTA and 1% SDS in 40 mM $NaHPO_4$, pH 7.2, all for 20 min at 65°C. For probing with β-actin, blots were prehybridized in Hybrisol I (Oncor, Gaithersberg, MD) for 60 min and hybridized overnight at 52°C. Blots were washed at 52°C in 0.1x SSC and 0.1% SDS. After a brief rinse in 1x SSC, the blots were exposed to Kodak XAR-2 X-ray film for 1 to 3 days. Quantitation was performed using a PhosphorImager 425 (Molecular Dynamics, Sunnyvale, CA).

The probe for the metabolic enzyme GAPDH was a 1.2-kb *Pst*I insert fragment from a plasmid containing the rat GAPDH cDNA. The β-actin cDNA probe was purchased from Oncor (Gaithersburg, MD). All probes were labeled with [α-$^{32}$P]dATP (6000 Ci/mmole; Dupont NEN, Boston, MA) using the Prime-It Random Priming Kit (Strategene, La Jolla, CA) to a specific activity of $10^9$ dpm/μg.

### Statistical Analysis

Student's *t*-test for independent values was used to test the significance of the differences for mean values of lung weights or pleural fluid volumes between vehicle-treated and methylprednisolone or 21-aminosteroid-treated rats. An extension of Fisher's exact test was used at each time point to test for significant differences in numbers of animals surviving among the four groups (vehicle-, methylprednisolone-, U74389G-, or U-74006F-treated) taken collectively. If this test was significant ($p < 0.05$) at a given time point, pairwise Fisher's exact tests were carried out to determine significant differences between the groups. Peto and Peto's log rank test (*32*) also was applied across time points to test for differences in the distributions of survival times among the four groups. Differences that produced *p*-values less than 0.05 were accepted as significant.

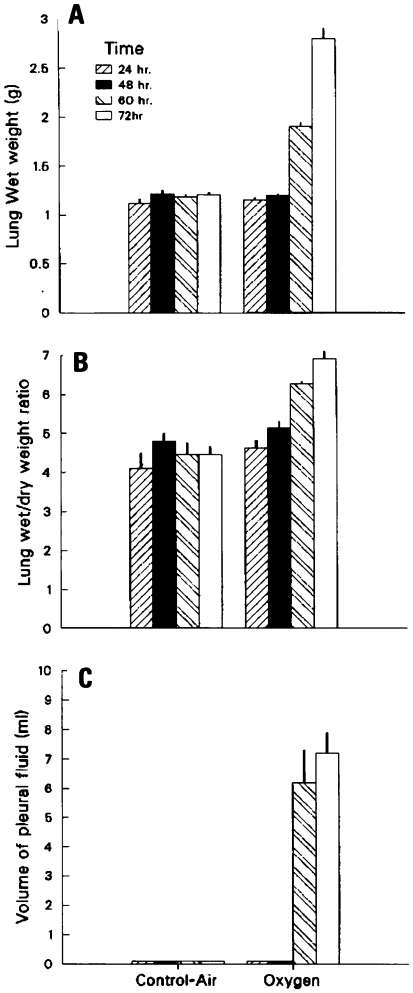


**Figure 2.** Time course of hyperoxic lung injury: data from air-breathing controls (left columns); >95% oxygen (right columns). (*A*) changes in lung wet weight; (*B*) lung wet weight to dry weight ratio; (*C*) volumes of pleural fluid recovered at 24, 48, 60, and 72 hr.

### Care and Use of Animals

All procedures in this study are in compliance with the Animal Welfare Act Regulations, 9CFR Parts 1, 2, and 3 and with the Guide for the Care and Use of Laboratory Animals, DHEW Publication (NIH) 85-23, 1985.

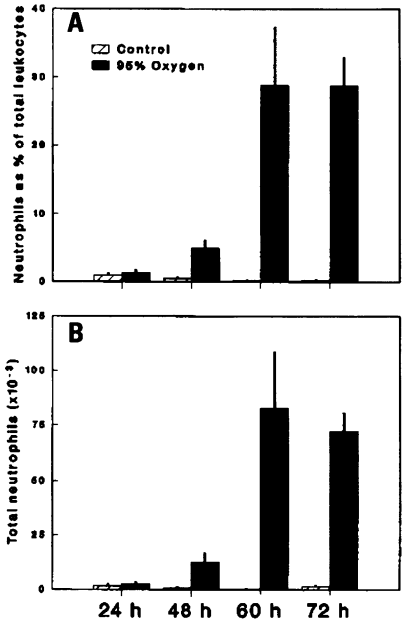


**Figure 3.** Time course of neutrophil infiltration into the airway lumen following inhalation of >95% oxygen for 24, 48, 60, and 72 hr. (*A*) neutrophils as a % of total cells recovered in bronchoalveolar lavage fluid. (*B*) absolute numbers of neutrophils recovered during hyperoxic lung injury. Hatched columns: air-breathing controls. Solid columns: >95% oxygen.

## Results

### Time Course of Hyperoxic Lung Injury, Neutrophil Influx, and Expression of P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in Hyperoxic Lungs

Exposure of rats to >95% oxygen for up to 72 hr induced a progressive decrease in body weight and increasing lung damage, demonstrated by a marked and significant ($p < 0.05$) increase in wet lung weight (Figure 2*A*), but no significant ($p > 0.05$) change in dry lung weight. Consequently, hyperoxia also induced a significant increase in the lung wet weight to dry weight ratio (Figure 2*B*). Hyperoxia also produced a marked and significant ($p < 0.05$) accumulation of fluid in the thorax at 60 hr after the initial exposure to >95% oxygen (Figure 2*C*). These changes were associated with a neutrophil-rich accumulation of inflammatory cells in the lungs (Figure 3*A,B*). Forty-eight hours after exposure to >95% oxygen there was a small but significant ($p < 0.05$) increase in the numbers of neutrophils recovered from BAL fluid. Sixty hours after exposure there was a marked infiltration of the bronchial lumen with inflammatory cells, neutrophils comprising approximately 30% of the total

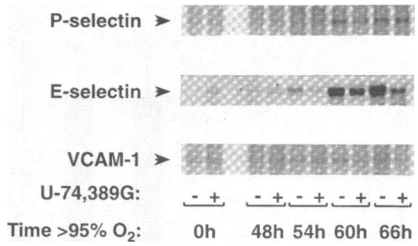


**Figure 4.** Northern blot analysis of mRNA for P-selectin, VCAM-1, and E-selectin before (0 hr) and 48, 54, 60, and 66 hr exposure to >95% oxygen. Lung tissues were taken from rats treated with U-74389G (30.0 mg/kg, ip) bid (+) or vehicle (–) throughout the period of oxygen exposure.

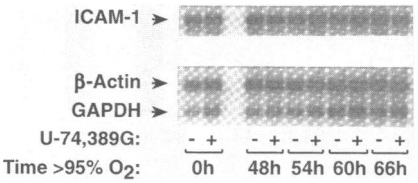


**Figure 5.** Northern blot analysis of mRNA for ICAM-1, before (0 hr) and 48, 54, 60, and 66 hr exposure to 95% oxygen. Blots were probed for GAPDH and β-actin.

cells in the BAL fluid (Figure 3*B*). The time course of neutrophil infiltration correlated with a marked upregulation of mRNAs for P-selectin and E-selectin, and a small upregulation of mRNA for VCAM-1 (Figure 4). ICAM-1 was constitutively expressed at a high level in both air-breathing and hyperoxic rats, and the levels did not change significantly during the progression of hyperoxic lung injury (Figure 5).

## Effect of Methylprednisolone, U-74389F, and U-74006F on Hyperoxic Lung Injury

Because some animals died after exposure to >95% oxygen for periods longer than 60 hr, we decided to evaluate compound activity in this model in animals exposed to >95% oxygen for 60 hr. At this time point all animals survived but there were marked and significant ($p < 0.05$) increases in lung weight and fluid accumulation in the thorax when compared with air-breathing controls. Pretreatment with 1, 3, and 10 mg/kg methylprednisolone, ip, 1 hr before oxygen exposure and then twice daily throughout the period of oxygen inhalation produced no significant ($p > 0.05$) effect on the hyperoxia-induced increases in lung wet weight (Figure 6 ) or pleural fluid accumulation (Figure 7). U-74006F, at all the dose levels tested and using the same dosing regimen as that for methylprednisolone, produced a marked and statistically significant ($p < 0.05$) inhibition of the oxygen-induced increases in pleural fluid, but not on the changes in lung weights. U-74389F also produced a significant ($p < 0.05$) inhibition of the oxygen-induced increases in pleural fluid at all doses and significantly inhibited ($p < 0.05$) the increases in lung weight at the 10 mg/kg, ip, dose level (Figures 6, 7).

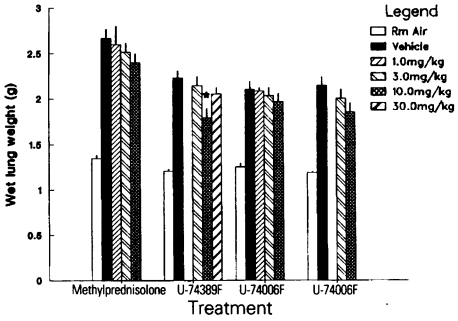


**Figure 6.** Effect of treatment with methylprednisolone and the 21-aminosteroids U-74389F and U-74006F on increases in lung weight induced by breathing >95% oxygen for 60 hr in Sprague-Dawley rats. Significant difference between vehicle and drug-treated animals, $^*p < 0.05$.

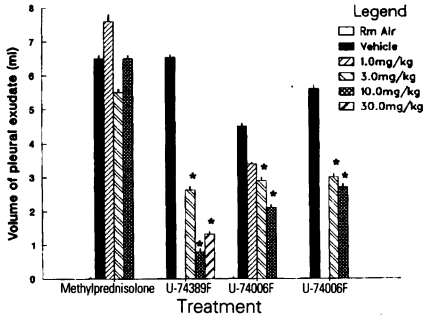


**Figure 7.** Effect of treatment with methylprednisolone and the 21-aminosteroids U-74389F and U-74006F on the accumulation of thoracic fluid in Sprague-Dawley rats breathing >95% oxygen for 60 hr. *Significant difference between vehicle and drug-treated animals, $p < 0.05$.

After demonstrating inhibition of hyperoxic lung injury with the 21-aminosteroids, but not with methylprednisolone, we wished to determine if these agents would influence the survival rates of rats exposed to >95% oxygen. When survival times were compared in groups of 12 animals exposed to >95% oxygen for up to 96 hr, we found that methylprednisolone 10-mg/kg, bid, treatment significantly ($p < 0.05$) decreased survival time, while both of the 21-aminosteroids (U-74389G and U-74006F) 10 mg/kg bid significantly ($p < 0.05$) increased survival times (via the logrank test as well as the Fisher's exact tests shown in Table 1). Under the experimental conditions described here, we found that exposure to 95% oxygen for 72 hr resulted in the death of approximately 50% of untreated rats or rats treated with

**Table 1.** Effect of methylprednisolone and the 21-aminosteroids U-74006F and U-74389G (10 mg/kg bid) on survival times of Sprague–Dawley rats exposed to >95% oxygen.

| | Time after $O_2$ exposure, hr[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 0 | 48 | 56 | 72 | 78 | 84 | 96 |
| Vehicle | 12/12 | 12/12 | 12/12 | 5/12 | 1/12 | 0/12 | 0 |
| Methylprenisolone | 12/12 | 12/12 | 9/12 | 0/12* | 0 | 0 | 0 |
| U-74006F | 12/12 | 12/12 | 12/12 | 6/12 | 5/12 | 5/12* | 4/12* |
| U-74389G | 12/12 | 12/12 | 12/12 | 8/12 | 7/12* | 5/12* | 5/12* |

[a]The data show the numbers of animals surviving/12. *Significant differences from vehicle-treated animals, $p < 0.05$. Note that the 21-aminosteroids significantly improved survival rates while methylprednisolone reduced survival rates.

**Table 2.** Effect of the 21-aminosteroid U-74389G (30 mg/kg bid) on the expression of mRNA for the adhesion molecules ICAM-1, VCAM-1, E-selectin, and P-selectin in rat lung tissues.[a]

| Time after $O_2$ exposure, hr | U-74389G | ICAM-1 | VCAM-1 | E-Selectin | P-Selection |
|---|---|---|---|---|---|
| 48 | – | 1.2 ± 0.1 | 1.3 ± 0.4 | 1.9 ± 1.3 | 1.1 ± 0.2 |
| | + | 1.8 ± 0.4 | 2.7 ± 0.9 | 3.1 ± 1.9 | 2.8 ± 2.2 |
| 54 | – | 1.4 ± 0.2 | 1.7 ± 0.8 | 2.6 ± 2.8 | 1.5 ± 0.6 |
| | + | 1.7 ± 0.5 | 2.0 ± 0.9 | 2.5 ± 1.0 | 1.8 ± 0.6 |
| 60 | – | 1.6 ± 0.4 | 1.8 ± 0.7 | 16.2 ± 9.0 | 10.5 ± 3.6 |
| | + | 1.4 ± 0.4 | 1.5 ± 0.6 | 8.4 ± 4.9 | 6.3 ± 2.8 |
| 66 | – | 1.8 ± 0.3 | 2.9 ± 2.2 | 12.6 ± 18.9 | 8.1 ± 2.7 |
| | + | 1.6 ± 0.3 | 1.5 ± 0.4 | 8.8 ± 2.7 | 12.8 ± 8.8 |

[a]The data show the fold increase in the levels of mRNA over the levels in air-breathing controls.

vehicle alone. All rats treated with methylprednisolone died by 72 hr, whereas approximately 50% of animals treated with the 21-aminosteroids survived to 96 hr.

We also explored the effects of treatment with one of the 21-aminosteroids, U-74389, (30.0 mg/kg, bid, ip) on the accumulation of neutrophils in the lung, and the expression of mRNA for P-selectin, E-selectin, ICAM-1, and VCAM-1 during the development of hyperoxic lung injury. We found that U-74389 did not significantly affect the numbers of neutrophils appearing in BAL fluid, nor did it significantly affect the levels of expression of mRNA for P-selectin, VCAM-1, ICAM-1, or E-selectin (Table 2).

## Discussion

It is now well established that prolonged oxygen therapy causes a progressive pulmonary edema and lung damage in all species studied so far, including humans (*1,33,34*). To date, there is no commonly accepted pharmacologic intervention for the treatment of this disorder and the mechanisms by which hyperoxia induces cell damage have not been fully elucidated. However, there is strong evidence that oxygen-derived free radicals (superoxide anion and hydroxyl radical), hydrogen peroxide, and peroxidation products of lipids play a significant role and therefore lead to an increase in capillary permeability and lung edema. Thus, studies in animal models of hyperoxic lung injury show that interventions which increase antioxidant enzymes correlate positively with increased survival (*35–39*), while those that compromise antioxidant defenses decrease survival (*40–44*). There are numerous other examples. Preexposure of rats to 85% oxygen induces tolerance to the effects of subsequent exposure to 100% oxygen and this is associated with an increase of lung levels of superoxide dismutase, an enzyme which converts $O_2^-$ to $H_2O_2$ (*35*). In accordance with these findings, treatments with superoxide dismutase or catalase have been shown to be protective against oxygen toxicity (*45,46*). In addition, hyperoxia also has been shown to increase oxygen radical production in rat lung homogenates (*47*), while oxygen metabolite scavengers inhibit hyperoxic lung injury (*48*) and protect alveolar macrophages from hyperoxic injury *in vitro* (*49*). Lipid peroxides also have been shown to be directly toxic to alveolar cells (*50*). The above findings support the view that hyperoxic lung injury occurs as a result of the natural antioxidant defenses being overwhelmed by the increased oxidant stress resulting from breathing high oxygen concentrations. Therapeutic interventions that might attenuate the oxidant stress would be of obvious value in this disorder.

Previously, we demonstrated an inhibition of hyperoxic lung injury after treatment with the nonglucocorticoid 21-aminosteroid U-74389F (*51*). 21-Aminosteroids were originally designed as inhibitors of iron-dependent lipid peroxidation (*15*) and are effective in animal models of CNS trauma and neuronal membrane damage (*16,17*). In the present study, we showed that the inhibitory properties of U-74389F on hyperoxic lung injury, as measured by the inhibition of the hyperoxia-induced accumulation of pleural fluid, was shared by a close structural analog, U-74006F (Tirilizad mesylate), a compound that is currently being evaluated for the treatment of head and spinal cord injury in humans. In contrast, methylprednisolone, when tested at the same dose levels and under the same dosing regimen, failed to demonstrate any protection of hyperoxic lung injury. Furthermore, when we compared survival times of rats exposed to >95% oxygen, methylprednisolone treatment reduced survival times, while the nonglucocorticoid 21-aminosteroids U-74389G and U-74006F significantly increased survival times. Although we have not explored the mechanisms by which methylprednisolone exacerbated oxygen toxicity, a similar decrease in survival times has been described by other investigators after glucocorticoid pretreatment of sheep (*52*), mice (*53*), and rats (*54*). Interestingly, the latter investigators showed that, although dexamethasone exacerbated lung damage and diminished survival when given early during exposure to hyperoxia, they were able to demonstrate an improved survival if given when the hyperoxia was soon to be terminated. In sheep, glucocorticoid treatment potentiated oxygen toxicity irrespective of the time of treatment (*52*). The abundance of experimental data, including our own, that suggests glucocorticoid treatment is deleterious to animals breathing high concentrations of oxygen may indicate that these agents should be avoided in clinical settings that require inhalation of high fractions of inspired oxygen. However, at the present time, high-dose steroids provide the only available pharmacologic treatment for patients with severe spinal cord injuries, and these patients often require ventilation with oxygen.

The effects of U-74389 and U-74006F in preventing hyperoxic lung injury and prolonging survival times in rats in our experiments are consistent with a recent report by Frank and McLauglin (*55*) that showed treatment of neonatal rats with U-74389F protected against the oxygen-induced inhibition of normal lung development. These investigators showed that the normal septation of the large air saccules at birth to form smaller diameter alveoli, with a much increased surface area, is markedly inhibited by hyperoxia. The model showed a very similar lung pathology to that which occurs in infants who had died with bronchopulmonary dysplasia. Treatment of 4-day-old rat pups for 10 days with U-74389F markedly reduced the oxygen-induced inhibition of normal lung development. Similarly, the oxygen-induced inhibition of elastin deposition, which is intimately involved in the septation process, was ameliorated by U-74389F treatment.

In addition to hyperoxic lung injury, 21-aminosteroids have also been shown to be efficacious in other models of pulmonary oxidant damage. Thus U-74500A, a close structural analog of the compounds tested here, has been shown to prevent oxidant-mediated injury in isolated rat lungs after ischemia/reperfusion or after *t*-butylhydroperoxide challenge (*19*). U-74389F also offers some protection against bleomycin-induced pulmonary fibrosis in rats (*56*). U-74500A and U-74389F also protect pulmonary endothelial cells against oxidant-dependent injury induced by neutrophils (*57*).

The mechanisms by which 21-aminosteroids inhibit hyperoxic lung injury are yet to be elucidated. However, these compounds are potent inhibitors of lipid peroxidation (*15,58*) and are highly lipophilic, distributing preferentially to the lipid bilayer of cell membranes where they have been shown to exert potent stabilizing effects (*59,60*). Consequently, it has been proposed (*61*) that these compounds exert their antilipid peroxidation activity through cooperative mechanisms, a radical scavenging action, and a physicochemical interaction with cell membranes that decreases membrane fluidity. This proposition is consistent with the compounds' effects on hyperoxic lung injury *in vivo*, where stabilization of endothelial cell membranes would reduce hyperoxia-induced increases in capillary permeability and pulmonary edema.

In our experiments, hyperoxic lung injury in rats was associated with a progressive increase in the numbers of neutrophils that could be washed out of the bronchial

lumen by lavage. This was consistent with findings from other laboratories in which increases in the numbers of radiolabeled neutrophils were detected during hyperoxic lung injury (*20*). To determine if there was a temporal association between the appearance of neutrophils in the bronchoalveolar lavage fluid and the expression of mRNA for the adhesion molecules, ICAM-1, E-selectin, VCAM-1, or P-selectin, we exposed rats to an atmosphere of >95% oxygen for up to 72 hr. Quantitation of mRNA for E-selectin and P-selectin in hyperoxic lung tissue demonstrated marked increases in both selectin molecules after prolonged exposure to oxygen, at 60 and 66 hr. This was temporally associated with the influx of neutrophils into the lungs and is consistent with the view that P-selectin expression on activated endothelial cells first promotes rolling of these cells (*22,25*) via interaction with neutrophil carbohydrate ligands (e.g., sialyl Lewis$^x$) presented by L-selectin, which is expressed at high levels on circulating resting neutrophils, and is constitutively functional (*21*). Following rolling of neutrophils, firm adhesion and transmigration through endothelial cells may then be mediated through the integrins LFA-1 and MAC-1 on the neutrophil surface with ICAM-1 on the endothelial cells. The increase in mRNA for E-selectin in hyperoxic lung tissue, and the associated neutrophilia seen in our experiments in rats, also is consistent with the observation that adhesion of neutrophils to endothelial cells can be mediated via L-selectin on the neutrophil surface with upregulated E-selectin on endothelial cells (*22*). This is in contrast to studies in mice (*62*) in which no upregulation of E-selectin mRNA was observed. Messenger RNA for ICAM-1 was constitutively expressed at high levels in air-breathing and hyperoxic rats, and no perceptible change was observed during the development of hyperoxic lung damage. This is in contrast to hyperoxic lung injury in murine lung tissue in which an increase in mRNA for ICAM-1 has been reported (*63*) and antiICAM-1 monoclonal antibody treatment partially inhibits hyperoxic lung injury (*64*). Signals for VCAM-1 were weak in rat lung tissues, but nonetheless, a slight increase in expression occurred at >54 hr of hyperoxia. The precise contribution made by each of the different adhesion molecules to leukocyte trafficking in hyperoxic lung injury may be best examined using deficient or mutant mice that lack the expression of P-selectin, CD18, and ICAM-1 (*65–67*).

When rats were pretreated with the 21-aminosteroid U-75389, we saw no significant inhibition of the neutrophil influx into bronchoalveolar lavage fluid and no significant inhibition of the bronchoalveolar neutrophilia or the expression of mRNA for the different adhesion molecules. We conclude from this that the effects of U-74389 cannot be ascribed to the inhibition of neutrophil trafficking into the lungs. The ability of 21-aminosteroids to prevent lung damage and promote survival of hyperoxic rats also adds further support to the view that neutrophil products do not contribute to the early (<60 hr) lung damage that occurs during exposure to very high concentrations of oxygen. Ibuprofen has been shown to inhibit neutrophil influx during hyperoxia in rabbits without preventing lung damage (*68*), and neutrophil depletion with cytotoxic drugs does not prevent acute hyperoxic injury (*69,70*). However it is likely that reactive oxygen metabolites from neutrophils serve to exacerbate lung damage during long-term exposure to high oxygen concentrations. It would be interesting to explore the effects of 21-aminosteroids in such a model.

The inhibition of hyperoxic lung damage by 21-aminosteroids, coupled with the improved survival seen in treated animals, suggests that 21-aminosteroids and their derivatives may be of potential clinical use in treating hyperoxic lung injury and other pulmonary conditions, such as ARDS, in which oxidant injury may contribute to the pathophysiology.

## REFERENCES


1. Deneke SM, Fanburg BL. Normobaric oxygen toxicity of the lungs. N Engl J Med 303:76–86 (1980).
2. Baldwin SR, Grum CM, Boxer LA, Simon RH, Ketai LH, Devall LJ. Oxidant activity in expired breath of patients with adult respiratory distress syndrome. Lancet 1:11–13 (1986).
3. Brigham KL, Meyrick B. Interactions of granulocytes with the lungs. Circ Res 54:623–635 (1984).
4. Heffner JE, Repine JE. Pulmonary strategies for antioxidant defense. Am Rev Respir Dis 1989:531–554 (1989).
5. Cantin AM, North SL, Fells GA, Hubbard RC, Crystal RG. Oxidant mediated epithelial cell injury in idiopathic pulmonary fibrosis. J Clin Invest 79:1665–1675 (1987).
6. Crystal RG, Ferrans VJ, Basset F. Biologic basis of pulmonary fibrosis. In: The Lung. Scientific Foundations (Crystal RG, West JB, eds). New York:Raven Press, 1991;2031–2046.
7. Roum JH, Buhl R, McElvaney N, Borok C, Chernick M, Caplan D, Crystal RG. *In vitro* evaluation of the ability of glutathione to suppress the inflammatory cell derived oxidant burden in respiratory epithelial lining fluid of individuals with cystic fibrosis. Clin Res 38:440A (1990).
8. Barton AD, Ryder K, Lourenco RV, Dralle W, Weiss SG. Inflammatory reaction and airway damage in cystic fibrosis. J Lab Clin Med 88:423–426 (1976).
9. Salh B, Webb K, Guyan PM, Day JP, Wickens D, Griffin J, Braganza JM, Dormandy TL. Aberrant free radical activity in cystic fibrosis. Clinical Chimica Acta 181:64–74 (1989).
10. Hubbard RC, Ogushi F, Fells GA, Cantin AM, Courtney M, Crystal RG. Oxidants spontaneously released by alveolar macrophages of cigarette smokers can inactivate the active site of α1-antitrypsin rendering it ineffective as an inhibitor of neutrophil elastase. J Clin Invest 80:1289–1295 (1987).
11. Hunninghake GW, Crystal RG. Cigarette smoking and lung destruction: accumulation of neutrophils in the lungs of cigarette smokers. Am Rev Respir Dis 128:833–838 (1983).
12. Barnes PJ. Reactive oxygen species and airway inflammation. Free Radic Biol Med 9:235–243 (1990).
13. Kanazawa H, Kurihara N, Hirata K, Takeda T. The role of free radicals in airway obstruction in asthmatic patients. Chest 100:1319–1322 (1991).
14. Jacobsen EJ, McCall JM, Ayer DE, Van Doornik FJ, Palmer JR, Belonga KL, Braughler JM, Hall ED, Houser DJ, Krook MA, Runge TA. Novel 21-aminosteroids that inhibit iron-dependent lipid peroxidation and protect against central nervous system trauma. J Med Chem 33(4):1145–1151 (1990).
15. Braughler JM, Pregenzer JF, Chase RL. A new 21-aminosteroid antioxidant lacking glucocorticoid activity stimulates ACTH secretion and blocks arachidonic release from mouse pituitary tumor (AtT-20) cells. J Pharmacol Exp Ther 244:423–427 (1988).
16. Hall ED, McCall JM, Chase RL, Yonkers PA, Braughler JM. A nonglucocorticoid steroid analog of methylprednisolone duplicates its high-dose pharmacology in models of central nervous system trauma and neuronal membrane damage. J Pharmacol Exp Ther 242:137–142 (1987).
17. Hall ED, Yonkers PA, McCall JM, Braughler JM. Effects of the 21-aminosteroid U-74006F on experimental head injury in

mice. J Neurosurg 68:456–461 (1988).
18. Richards IM, Shields SK, Griffin RL, Fidler SF and Dunn CJ. Novel steroid-based inhibitors of lung inflammation. Clin Exp Allergy 22:432–439 (1992).
19. Haynes J, Seibert A, Bass JB, Taylor AE. U-74500A inhibition of oxidant mediated lung injury. Am J Physiol 259:H144–H148 (1970).
20. Rinaldo JE, English D, Levin J, Stiller R, Henson J. Increased intrapulmonary retention of radiolabeled neutrophils in early oxygen toxicity. Am Rev Respir Dis 137:345–352 (1988).
21. Butcher DC. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 67:1033–1036 (1991).
22. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell 65:859–873 (1991).
23. Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. J Cell Biol 115:223–234 (1991).
24. Bevilacqua MP, Stengelin S Jr, Gimbrone MA, Seed B. Endothelial-leukocyte adhesion molecule-1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 243:1160–1165 (1989).
25. Picker LR, Warnock RA, Burns AR, Doerschuk CM, Berg EL, and Butcher EC. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. Cell 66:921–933 (1991).
26. Paulson JC. Selectin/carbohydrate-mediated adhesion of leukocytes. In: Adhesion: Its Role in Inflammatory Disease (Harlan JM, Liu DY, eds). New York:WH Freeman & Co, 1992; 19–42.
27. Montefort S, Roche WR, Howarth PH, Djukanovic R, Gratziou C, Carroll M, Smith L, Britten KM, Haskard D, Lee TH, Holgate ST. Intercellular adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1) expression in the bronchial mucosa of normal and asthmatic subjects. Eur Respir J 5:815–823 (1992).
28. Georas SN, Liu MC, Newman W, Beall LD, Stealey BA, and Bochner BS. Altered adhesion molecule expression and endothelial cell activation accompany the recruitment of human granulocytes to the lung after segmental antigen challenge. Am J Respir Cell Mol Biol 7:261–269 (1992).
29. Kita Y, Takashi T, Iigo Y, Tamatani T, Miyasaka M, Horiuchi T. Sequence and expression of rat ICAM-1. Biochem Biophys Acta 1131:108–110 (1992).
30. Hession C, Moy P, Tizard R, Chisholm P, Williams C, Wysk M, Burkly L, Miyake K, Kincade P, Lobb R. Cloning of murine and rat vascular cell adhesion molecule-1. Biochem Biophys Res Comm 183:163–169 (1992).
31. Krzesicki RF, Hatfield CA, Bienkowski MJ, McGuire JC, Winterrowd GE, Chapman DL, Berger AE, McEwan RN, Carter DB, Chosay JG, Tracey DE, Chin JE. Regulation of expression of IL-1 receptor antagonist protein in human synovial and dermal fibroblasts. J Immunol 150:4008–4018 (1993).
32. Peto R, Peto J. Asympototically efficient rank invariant test procedures. J Roy Stat Soc Ser A 135:185–206 (1972).
33. Durr RA, Thet LA. Animal models of hyperoxic lung injury. In: CRC Handbook of Animal Models of Pulmonary Disease, Vol 1 (Cantor J, ed). Boca Raton, FL:CRC Press, 1989;3–17.
34. Forman HJ. Oxidant radical production and lung injury. In: Oxygen Radicals: Systemic Events and Disease Processes (Das DK, Essman WB, eds). Basel:Karger 1990;71–96.
35. Crapo JD, Tierney DF. Superoxide dismutase and pulmonary oxygen toxicity. Am J Physiol 226:1401–1407 (1974).
36. Crapo JD, Barry BE, Foscue HA. Structural and biochemical changes in rat lungs occurring during exposures to lethal and adaptive doses of oxygen. Am Rev Respir Dis 122:123–43 (1980).
37. Forman HJ, Fisher AB. Antioxidant enzymes in rat granular pneumocytes: constitutive levels and effect of hyperoxia. Lab Invest 45:1–6 (1981).
38. Frank L, Yam J, Roberts RJ. The role of endotoxin in protection of adult rats from oxygen-induced lung toxicity. J Clin Invest 61:269–275 (1978).
39. Frank L, Summerville J, Massaro D. Protection from oxygen toxicity with endotoxin. Role of the endogenous antioxidant enzymes of the lung. J Clin Invest 65:1104–1110 (1980).
40. Cross CE, Hasegawa G, Reddy KA, et al. Enhanced lung toxicity of $O_2$ in selenium-deficient rats. Res Commun Chem Pathol Pharmacol 16:695–706 (1977).
41. Deneke SM, Bernstein SP, Fanburg BL. Enhancement bydisulfiram (antabuse) of toxic effects of 95 to 97% $O_2$ on the rat lung. J Pharmacol Exp Ther 208:377–780 (1978).
42. Deneke SM, Gershoff SN, Fanburg BL. Potentiation of oxygen toxicity in rats by dietary protein or amino acid deficiency. J Appl Physiol 54:147–151 (1983).
43. Forman HJ, York JL, Fisher AB. Mechanism for the potentiation of oxygen toxicity by disulfiram. J Pharmacol Exp Ther 212:452–455 (1980).
44. Forman HJ, Rotman EI, Fisher AB. The roles of selenium and sulfur-containing amino acids in protection against oxygen toxicity. Lab Invest 49:148–153 (1983).
45. McLennan G, Autor AP. Effect of intraperitoneally administered superoxide dismutase on pulmonary damage resulting from hyperoxia. In: Pathology of Oxygen (Autor AP, ed). New York:Academic Press, 1982;85.
46. Turrens JF, Crapo JD, Freeman BA. Protection against oxygen toxicity in intravenous injection of liposome-entrapped catalase and superoxide dismutase. J Clin Invest 73:87–95 (1984).
47. Freeman BA, Young SL, Crapo JD. Liposome-mediated augmentation of superoxide dismutase in endothelial cells prevents oxygen injury. J Biol Chem 258:12534–12542 (1983).
48. Beehler CJ, Simchuk ML, McCord JM, Repine JE. Effects of dimethylthiourea in hyperoxic lung injury. J Lab Clin Med 119:508–513 (1992).
49. Harada RN, Vatter AE, Repine JE. Oxygen radical scavengers protect alveolar macrophages from hyperoxic injury *in vitro*. Am Rev Respir Dis 128:761–772 (1983).
50. Ozawa T, Hayakawa M, Takamura T, Sugiyama S, Suzuki K, Iwata M, Taki F, Tami T. Biosynthesis of leukotoxin 9,10-epoxy-12-octadecanoate, by leukocytes in lung lavages if rat after exposure to hyperoxia. Biochem Biophys Res Commun 134:1071 (1986).
51. Richards IM, Griffin RL, Fidler SF, Jacobsen EJ. Effect of the 21-aminosteroid U-74389F on hyperoxic lung injury in rats. Proceedings of the Sixth International Conference of the Inflammation Research Association, September 20–24, 1992, White Haven, PA. Agents Actions 39:C136–138 (1993).
52. Newman JH, Fulkerson WJ, Kobayashi T, English D, Meyrick B, Brigham, KL. Effects of methylprednisolone on lung oxygen toxicity in awake sheep. J Appl Physiol 60:1386–1392 (1986).
53. Gross NJ, Smith DM. Methylprednisolone increases toxicity of oxygen in adult mice. Mechanical and biochemical effects of the surfactant system. Am Rev Respir Dis 129:805–810 (1984).
54. Koizumi M, Frank L, Massaro D. Oxygen toxicity in rats. Varied effects of dexamethasone treatment depending on duration of hyperoxia. Am Rev Respir Dis 131:907–911 (1985).
55. Frank L, McLaughlan GE. Protection of acute and chronic hyperoxic inhibition of neonatal rat lung development with the 21-aminosteroid drug U74389F. Pediatr Res 33:632–638 (1993).
56. McLaughlin GE, Frank L. *In vivo* protective effects of the new 21-aminosteroid U-74389F against bleomycin-induced pulmonary fibrosis in rats. Am Rev Respir Dis 145:A574 (1992).
57. Meyer RJ, Juarez RA, Holden WE. 21-Aminosteroids protect endothelial cells against injury by neutrophils. Am Rev Respir Dis 145:A571 (1992).
58. Braughler JM, Pregenzer JF. The 21-aminosteroid inhibitors of lipid peroxidation: reactions with lipid peroxyl and phenoxy radicals. Free Radic Biol Med 7:125–130 (1989).
59. Audus KL, Guillot FL, Braughler JM. Evidence for 21-aminosteroid association with the hydrophobic domains of brain

microvessel endothelial cells. Free Radic Biol Med 11:361–371 (1992).
60. Hinzmann WE, McKenna RL, Pierson TS, Han F, Kezdy FJ, Epps DE. Interaction of antioxidants with depth-dependent fluorescence quenchers and energy transfer probes in lipid bilayers. Chem Phys Lipids 62:123–138.
61. Hall ED, McCall JM, Means ED. Therapeutic potential of the lazaroids (21-aminsteroids) in acute CNS trauma, ischemia and subarachinoiud hemorrage. Adv Pharmacol (in press).
62. Zeb T, Welty SE, Rivera JL, Ballantyne CM, Hanse TN. mRNA for E-selectin and vascular adhesion molecule (VCAM) are not increased in hyperoxic mouse lungs. FASEB J 7:A785 (1993).
63. Welty SE, Rivera JL, Elliston JF, Smith CV, Zeb T, Ballantyne CM, Montgomery CA, Hansen TN. Increases in lung tissue expression of intercellular adhesion molecule-1 are associated with hyperoxic lung injury and inflammation in mice. Am J Respir Cell Mol Biol 9:393–400 (1993).
64. Wegner CD, Wolyniec WW, LaPlante AM, Marschman K, Lubbe K, Haynes N, Rothlein R, Letts LG. Intercellular adhesion molecule-1 contributes to pulmonary oxygen toxicity in mice: role of leukocytes revised. Lung 170:267–279 (1992).
65. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. Cell 74:541–554 (1993).
66. Wilson RW, Ballantyne CM, Smith CW, Montgomery C, Bradley A, O'Brien WE, Beaudet AL. Gene targeting yields a CD18-mutant mouse for study of inflammation. J Immunol 151:1571–1578 (1993).
67. Sligh JE Jr, Ballantyne CM, Rich SS, Hawkins HK, Smith CW, Bradley A, Beaudet AL. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. Proc Natl Acad Sci USA 90:8529–8533 (1993).
68. Das DK, Bandyopadhyay D, Hoory S, Steinberg H. Role of polymorphonuclear leukocytes in hyperoxic lung injury. Prevention of neutrophil influx into the lung endothelium during oxygen exposure by ibuprofen. Biomed Biochim Acta 47:1023–1036 (1988).
69. Boyce NW, Campbell D, Holdsworth SR. Granulocyte independence of pulmonary oxygen toxicity in the rat. Exp Lung Res 15:491–498 (1989).
70. Laughlin MJ, Wild L, Nickerson PA, Matalon S. Effects of hyperoxia on alveolar permeability of neutropenic rabbits. J Appl Physiol 61:1126–1131 (1986).